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PCT

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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	**U.S. APPLICATION NO. (If known, see 37 CFR 1.5)** (Not Yet Assigned - U.S. National Phase of Int'l PCT No. PCT/IB99/01232 filed June 30, 1999) 09/720923

INTERNATIONAL APPLICATION NO. PCT/IB99/01232	INTERNATIONAL FILING DATE June 30, 1999	PRIORITY DATE CLAIMED July 1, 1998

TITLE OF INVENTION: NOVEL CYCLOSPORIN HAVING AN IMPROVED ACTIVITY PROFILE

APPLICANT(S) FOR DO/EO/US Roland M. WENGER; Manfred MUTTER; and Thomas RUCKLE

Applicant herewith submits to the United States Designated/Elected Office(DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.56, 1.97 and 1.98 with PTO Form 1449 and the International Search Report attached.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
    ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

PCT International Application Published Under the Patent Cooperation Treaty;
PCT International Search Report (Form PCT/ISA/210);
PCT Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/409);
PCT Request (Form PCT/RO/101).

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Search report has been prepared by the EPO or JPO . . . . . $ 860.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) $

No international preliminary examination fee paid to USPTO (37 CFR 1.482 but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . . $

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . . $

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(2)-(4) . . . . . $

			ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$130.00	
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**		
Total Claims	19 -20 =	0	X $18.00	$0.00	
Independent Claims	1 - 3 =		X $80.00	$0.00	
Multiple dependent claims(s) (if applicable)	0		+ $270.00	$0.00	
			TOTAL OF ABOVE CALCULATIONS =	$990.00	
Applicant qualifies for small entity status. See C.F.R. 1.27. The fees indicated above are reduced by 1/2.				$	
			SUBTOTAL =	**$990.00**	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$0.00	
			TOTAL NATIONAL FEE =	**$990.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31).					
			$ 40.00 per property +	$	
			TOTAL FEES ENCLOSED =	**$990.00**	
				Amount to be:	
				refunded	$
				charged	$.00

a. ☒ A check in the amount of $ 990.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 08-1650 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-1650.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Michael O. Sturm

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IN THE UNITED STATES TRADEMARK OFFICE

Applicants : WENGER Roland, MUTTER Munfred & RUCKLE Thomas

Serial n° (Not assigned yet) (Which is issued from PCT IB99/01232, filed June 30, 1999 by DEBIOPHARM S. A., entitled **NOVEL CYCLOSPORIN HAVING AN IMPROVED ACTIVITY PROFILE**, and is incorporated herein by reference.

Filed : On even Date Herewith

FOR: **NOVEL CYCLOSPORIN HAVING AN IMPROVED ACTIVITY PROFILE**

PRELIMINARY AMENDMENT

Assistant Commisionner for Patents
Washington, D.C. 20231

Dear Sir or Madam:

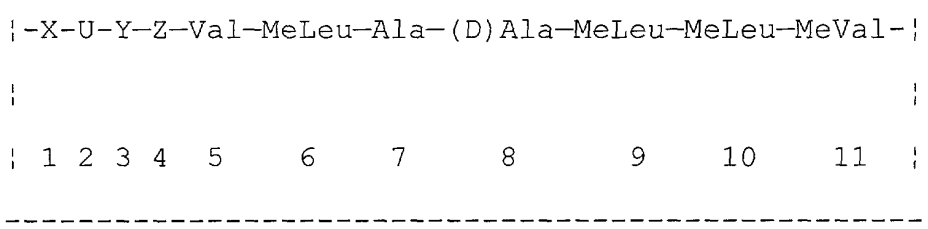
Prior to the calculation of fees and the examination of the above-identified application, kindly amend the application as follows :

AMENDMENT

Please kindly cancel claims 1 to 7 and substitute therefor new claims 8 to 14.

Claims

8. Synthesised cyclosporin having the formula:



wherein:

X is -MeBmt or 6,7-dihydro-MeBmt-

U is -Abu, Nva, Val, Thr

Y is Sar or (D)-MeSer or (D)-MeAla or (D)-MeSer (OAcyl)

Z is (N-R)aa where aa={Val, Ile, Thr, Phe, Tyr, Thr (OAc), Thr (OG_1), Phe (G_2), PheCH_2(G_3), Tyr (OG_3)} with R = {alkyl > CH_3};

G_1 = {phenyl-COOH, phenyl-COOMe, phenyl-COOEt};

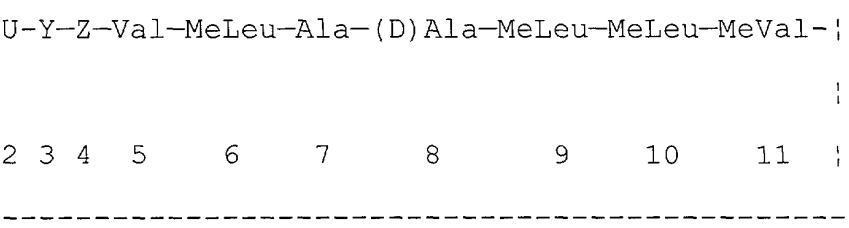
G_2 = {CH_2COOH, $CH_2COOMe(Et)_4$; $CH_2PO(OMe)_2$, $CH_2PO(OH)_2$};

G_3 = {$PO(OH)_2$, $PO(OCH_2CH{=}CH_2)_2$, CH_2COOH, $CH_2COOMe(Et)$}.

9. Cyclosporin according to claim 8, wherein the residue Z in position 4 is (R)Val where R>CH_3 and R<$C_{10}H_{21}$.

10. Cyclosporin according to claim 8, wherein the residue Z in position 4 is N-ethyl-valine.

11. Pharmaceutical composition containing the compound having the formula:



wherein:

X is -MeBmt or 6,7-dihydro-MeBmt-

U is -Abu, Nva, Val, Thr

Y is Sar or (D)-MeSer or (D)-MeAla or (D)-MeSer (OAcyl)

Z is (N-R)aa where aa={Val, Ile, Thr, Phe, Tyr, Thr (OAc), Thr (OG_1), Phe (G_2), PheCH_2(G_3), Tyr (OG_3)} with R = {alkyl > CH_3};

G_1 = {phenyl-COOH, phenyl-COOMe, phenyl-COOEt};

G_2 = {CH_2COOH, $CH_2COOMe(Et)$, $CH_2PO(OMe)_2$, $CH_2PO(OH)_2$};

G_3 = {$PO(OH)_2$, $PO(OCH_2CH=CH_2)_2$, CH_2COOH, $CH_2COOMe(Et)$}

12. Pharmaceutical composition according to claim 11, wherein it is combined with a pharmaceutically acceptable solution.

13. A medicinal product intended for the treatment and prevention of AIDS, containing the cyclosporin according to claim 8 or claim 11.

14. A medicinal product intended for the treatment and prevention of AIDS containing the cyclosporin according to claim 10.

Respectfully submitted,

Roland M. WENGER; Manfred MUTTER; and
Thomas RUCKLE

Dec. 28, 2000
Date

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IN THE MATTER OF INTERNATIONAL PATENT APPLICATION No. PCT IB99/01232 FILED ON JUNE 30, 1999 IN THE NAME OF DEBIOPHARM S.A.

and

IN THE MATTER OF AN APPLICATION FOR A PATENT IN UNITED STATES CORRESPONDING THERETO

VERIFICATION OF ENGLISH TRANSLATION

OF INTERNATIONAL APPLICATION

I, Cyra NARGOLWALLA of CABINET PLASSERAUD - 84 rue d'Amsterdam, 75009 PARIS, France, declare that I am well acquainted with the English and French languages and that the English translation, submitted herewith, of the above-identified International Application, which was filed in France, is a true and accurate translation.

Date: December 19, 2000

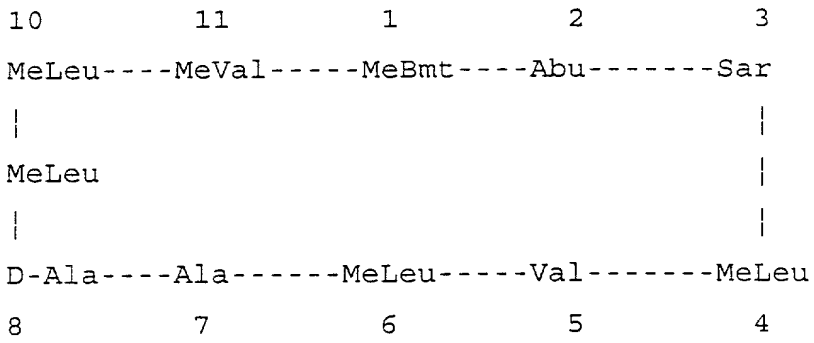
Signature: 

Novel cyclosporin having an improved activity profile

The present invention relates to a novel cyclosporin (Cs), the pharmaceutical use thereof and to a pharmaceutical composition containing it.

Cs are a class of cyclic poly-N-methylated undecapeptides having several pharmacological activities; in particular, they are immunosuppressants, anti-inflammatories, anti-parasitic agents, drug resistance suppressors (anti-MDR) and anti-viral agents. The first cyclosporin isolated from a fungal culture is cyclosporin A which is found in the natural state and which is represented by the following formula:

Structure of cyclosporin A



Abu = L-α-aminobutyric acid

Ala = L-alanine

MeBmt = N-methyl-(4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine

Leu	=	L-leucine
MeLeu	=	N-methyl-L-leucine
MeVal	=	N-methyl-L-valine
Nva	=	L-norvaline
Sar	=	sarcosine
Thr	=	L-threonine
Val	=	L-valine

The amino acids described according to their conventional abbreviation have the configuration L unless otherwise specified.

Since this first cyclosporin was discovered, a large number of other varieties have been isolated and identified, as have non-natural varieties obtained by synthetic or semi-synthetic methods, or even by the application of modified culture techniques. The production of cyclosporin A is described by [Kobel et al. European Journal of Applied Microbiology and Biotechnology 14, 237-240 (1982)]. The production of artificial cyclosporins produced by a purely synthetic method developed by R. Wenger is also described - see Traber et al. 1, Traber et al. 2 and Kobel et al., US 4,108,985; 4,210,581; 4,220,641; 4,288,431; 4,554,351 and 4,396,542; EP 34 567 and 54 782; WO 86/02080; Wenger 1, Transpl. Proc. 15, Suppl. 1:2230 (1983); Wenger 2, Angew. Chem.Int. Ed., 24,77 (1985); and Wenger 3, Progress in the Chemistry of Organic Natural Products 50, 123 (1986).

Cyclosporin A (CsA) isolated 20 years ago from *Tolypocladium inflatum* has considerable immunosuppressive activity. It has revolutionised organ transplantation and is commonly used in the treatment of autoimmune diseases. For a recent review of the use of CsA and its mechanisms of action, see Wenger et al: Cyclosporine Chemistry, Structure-activity relationships and Mode of Action, Progress in Clinical Biochemistry and Medicine, Vol. 2, 176 (1986).

The therapeutic effect of CsA results mainly in the selective suppression of the activation of T lymphocytes. This immunosuppressive activity is explained by the fact that CsA binds to an intracellular proteic receptor, cyclophilin A (CyP) forming a CyP-CsA complex which interacts with calcineurin (CaN) and thus inhibits its phosphatase activity. Thus, the transcription of families of genes exhibiting precocious activation will be blocked (cf. O'Keefe, S.J; Tamura, J; *Nature* 1992, 357, 692-694).

The present invention provides the production of a novel cyclosporin with considerable HIV-1 (human immunodeficiency virus) inhibitory activity and not having the immunosuppressive activity of CsA.

The mode of infection of HIV type 1 is unique amongst the retroviruses because it requires the specific incorporation into its virion of the cellular protein CyP which interacts with the polyprotein Gag (cf. Eltaly Kara Franke, Bi-Xing Chem. Journal of Virology, Sept. 1995, vol. 69 no. 9). It is well known that CyP binds to CsA and CaN in

a ternary complex. However, the native function of CyP is to catalyse the isomerisation of peptidyl-prolyl bonds, a limiting and important step in the process allowing proteins to acquire a definitive three-dimensional structure. CyP also protects cells from thermal shocks or acts as a chaperone protein. Unlike CsA, the product of the Gag gene of HIV-1 prohibits the formation of a ternary complex with CyP and CaN. In fact, the HIV virus needs CyP in order to bind to the product of the Gag gene so as to form its own virions (cf. Franke, E.K; 1994 *Nature* 372, 359-362). In the presence of CsA, there is direct competition with the polyprotein derived from the Gag gene of HIV-1 to bind to CyP. This CsA acts at two levels on the replication of the viral cycle. Firstly, at the level of translocation towards the core of the pre-integrated complex, then in the production of infectious viral particles.

US patent 4,814,323 already describes anti-HIV activity of CsA, but the latter also has considerable immunosuppressive activity which is undesirable for the treatment of patients infected with the HIV virus. Recently, another type of cyclosporin has been developed, namely derivatives in position 4 such as MeIle^{4}Cs, MeVal^{4}Cs, or (4-OH)MeLeu^4-Cs to mention only the most anti-HIV and the least immunosuppressive substances. The derivative [(4-OH) MeLeu^4-Cs] is synthesised by oxidation of cyclosporin A using a microorganism. Another patent WO 97/04005 uses the method of preparation of the patent EP 484 281 and the method developed by Seebach EP 194972 in order to produce

derivatives in position 3 such as, for example, (D)-MeSer^3-(4-OH)MeLeu^4 cyclosporin. This substance has a better affinity for CyP but only has limited anti-HIV activity compared with the reference derivative MeIle^4-Cs(NIM 811). The more hydrophilic nature of this substance prevents it penetrating the cells and the organism. This is reflected directly in the reduced anti-HIV activity of this substance (cf. Christos Papageorgiou, J.J. Sanglier and René Traber - *Bioorganic & Medicinal Chemistry Letters*, Vol, 6, No. 1, pp. 23-26, 1996).

The substances described in this invention have the dual advantage of retaining the same affinity for CyP as that observed with [(4-OH)MeLeu^4]-Cs or cyclosporin A whilst having anti-HIV activity which is identical to or greater than that of the reference derivatives (MeVal^4-Cs or MeIle^4-Cs) and appreciably greater than the anti-HIV activity of cyclosporin A or of (4-OH)MeLeu^4-Cs. The object of the invention is to provide a novel cyclosporin which does not have the immunosuppressive activity of CsA and has an improved profile of activity. This new family of Cs is characterised by the formula (I):

```
|-X-U-Y-Z-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-|
|                                                 |
|1 2 3 4  5    6    7     8      9    10    11    |
---------------------------------------------------
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(I)

wherein:

X is -MeBmt or 6,7-dihydro-MeBmt-

U is -Abu, Nva, Val, Thr

Y is Sar or (D)-MeSer or (D)-MeAla or (D)-MeSer (OAcyl)

Z is (N-R)aa where aa={Val, Ile, Thr, Phe, Tyr, Thr (OAc), Thr (OG_1), Phe (G_2), PheCH_2(G_3), Tyr (OG_3)} with R = {alkyl > CH_3};

G_1 = {phenyl-COOH, phenyl-COOMe, phenyl-COOEt};

G_2 = {CH_2COOH, $CH_2COOMe(Et)$, $CH_2PO(OMe)_2$, $CH_2PO(OH)_2$};

G_3 = {$PO(OH)_2$, $PO(OCH_2CH=CH_2)_2$, CH_2COOH, $CH_2COOMe(Et)$}

Thus, by replacing the natural MeLeu group in position 4 by an N-(alkyl)aa group (where alkyl > CH_3), the anti-HIV 1 activity of this derivative is improved.

The new cyclosporin molecule thus obtained offers the unexpected and surprising advantage of having much better stability towards metabolisation than all the other cyclosporins known hitherto.

This new molecule is much more resistant to the phenomena of oxidation and degradation which take place in the cell. Consequently, the "in vivo" life of this new N-alkyl aa Cs is particularly prolonged.

Moreover, this new N-alkyl aa^4 cyclosporin has high affinity for CyP and has anti-HIV activity which is equal to or greater than the best existing cyclosporins.

Figure 1 represents the general structure of this novel cyclosporin. The groups R1, R2, R3 and R4 will be largely described in Table III. Thus, by transforming these 4 key positions, it was possible to retain a very good affinity for cyclophilin and to prevent the formation of a ternary complex with CaN and, above all, to increase, in a particularly advantageous manner, its stability towards enzymatic oxidation and consequently its anti-HIV activity.

This novel cyclosporin is thus characterised principally by the presence, in position 4, of a residue with $R>CH_3$ and $R < C_{10}H_{21}$. The substituent of nitrogen used will be, for example, ethyl, propyl, butyl or pentyl, but these examples are not limiting. This novel cyclosporin is particularly active if the residue in position 4 is an N-ethylated amino acid (see drawings 2 and 3).

The invention also claims the pharmaceutical composition of the substance as described by formula (I). This may be combined with a pharmaceutically acceptable solution. The pharmaceutical formulation thus produced makes it possible to increase the solubility in water or to keep the composition in the form of microemulsions in suspension in water. The object of the present invention is also to provide a new medicinal product which may be used, for example, in the treatment and prevention of AIDS (acquired immunodeficiency syndrome). The cyclosporin modified in position 4 by a residue Z, namely N-ethyl-valine will be used in particular for the production of a medicinal product

intended for the treatment and prevention of AIDS. The application for the prevention of AIDS is not limiting. This substance may also be used, for example, for its anti-inflammatory properties.

With regard to the process for the production of this novel cyclosporin, we used conventional techniques described in the literature and certain specific methods developed in the laboratory.

The process for the synthesis of CsA is described in: R. Wenger (Helv. Chim. Acta Vol. 67, p. 502-525 (1984)). The process for opening protected cyclosporin A (OAc) is described in Peptides 1996. The CsA molecule is treated with Meerwein's reagent $(CH_3)_3OBF_4$ then cleaved by treatment with acid in methanol or hydrolysed by water in order to convert it to a linear peptide of 11 amino acid residues: H-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OCH3. This process was presented at the international conference of the European Society of Peptides (EPS-24) in Edinburgh 8-13 September 1996 and published in Peptides 1996 by R. Wenger. This linear peptide is then treated according to the conventional Edman process in order to cleave its last amino acid residue (MeLeu) and to provide our starting product: the decapeptide H-Val-MeLeu-Ala-(D)Ala-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OMe. This product is then used in the following steps:

Preparation of (1) (protection):

Boc-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeValMeBmt(OAc)-Abu-Sar-OMe (1)

0.72 ml (4.18 mmoles) of a solution of diisopropylethylamine and 0.65 g (2.95 mmoles) of Boc anhydride in 50 ml of dioxane are added to a solution of 2.83 g (2.46 mmoles) of the decapeptide H-Val MeLeu-Ala-(D)Ala-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OMe in 120 ml of dioxane. 17 ml of water are added to the solution which is mixed for 2 hours at ambient temperature. The solvent is then evaporated and the resulting reaction mixture is dissolved in 300 ml of ethyl acetate then washed 3 x with a 5% solution of citric acid, 3 x with a saturated solution of $NaHCO_3$ and finally 3 x with a solution of NaCl. The organic phases are dried with anhydrous Na_2SO_4, filtered, and the solvent is finally evaporated under vacuum. 3 g (98%) of the protected decapeptide (Boc-decapeptide methyl ester) are thus obtained.

The product is then used for the following synthesis routes without an additional purification step. This substance is hydrolysed then activated and condensed with 1 corresponding amino acid in order to produce a new peptide with 11 residues, the starting product for the cyclisation and production of a novel cyclosporin with the desired properties.

Preparation of (2) (hydrolysis of the ester):

Boc-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeValMeBmt(OAc)-Abu-Sar-OH (2)

192 mg (4.56 mmoles) of $LiOH/H_2O$ dissolved in 36 ml of water are added dropwise (at 15°C) to 4.08 g (3.26 mmoles) of the previous compound (1) in 146 ml of tetrahydrofuran. The whole mixture is stirred at 15°C. The reaction is complete after 120 hours after the successive addition of 5 portions respectively of 1.4 equivalents of $LiOH/H_2O$ each. The solution obtained is neutralised with 0.1 N HCl and the solvent is then evaporated. The solid product recovered is then dissolved in 500 ml of ethyl acetate and washed 2 x with a 5% solution of citric acid and 2 x with a brine solution. The aqueous phases are extracted 4 x with 50 ml of ethyl acetate and the combined organic phases are then dried with anhydrous Na_2SO_4, filtered and evaporated. 3.84 g (95%) of compound (2) are thus obtained. The product is then used without additional purification.

Preparation of (3) (addition of a new amino acid):

Boc-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar—EtVal-OtBu (3)

6.18 g (5 mmoles) of compound (2) are dissolved in 250 ml of anhydrous dichloromethane under argon. The solution is then cooled and 3.9 ml of N-methylmorpholine (10 mmoles; pH 8.5) and 1.1 ml (10 mmoles) of isobutylchloroformate are then added slowly under argon. The solution is stirred for

15 minutes at -15°C. A solution of 2.4 g (12 mmoles) of H-NEt Val-OtBu dissolved in 40 ml of anhydrous dichloromethane is then added within a period of 20 minutes. The mixture is then stirred for 1 hour at -15°C, then for 1 hour at 0°C and finally overnight at ambient temperature. 400 ml of dichloromethane are then added, then 3 extractions are carried out with a 5% solution of citric acid followed by 3 extractions with a saturated solution of $NaHCO_3$ and finally 3 final extractions with a saturated solution of NaCl. The organic phases are dried with anhydrous Na_2SO_4 then filtered and finally the solvent is evaporated. After chromatography, 4.42 g (62%) of pure undecapeptide are recovered.

Preparation of (4) (deprotection):

H-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-EtVal-OH (4)

830 mg (0.58 mmole) of protected undecapeptide (4) are dissolved in 15 ml of pure dichloromethane. 3.2 ml of trifluoroacetic acid are added to this solution within a period of 3 minutes at ambient temperature. The reaction is monitored by HPLC which proves to be complete after 1 h 30. The solvent is evaporated and the remaining trifluoroacetic acid is evaporated 2 x in the presence of ethyl acetate.

The crude product (900 mg) is purified by chromatography [150 g of silica gel (0.4-0.63)], use of dichloromethane/methanol/triethylamine (17:3:0.05) as

eluants) to elute 700 mg (95%) of pure, deprotected undecapeptide (4).

Preparation of (5) (cyclisation):

MeBmt(OAc)^1-EtVal^4-Cs (5)

275 mg of TFFH (1.04 mmoles) are dissolved under argon in 3.45 l of anhydrous dichloromethane. The deprotected undecapeptide (4) [438 mg (0.347 mmole)] is then dissolved in 40 ml of anhydrous dichloromethane, and 0.52 ml (3.82 mmoles) of collidine are added thereto. This slightly basic peptide solution is added dropwise to the solution of TFFH within a period of 20 minutes under argon and with vigorous stirring. After 1 h 30 all the starting material is cyclised. In order to trap the excess TFFH, 5 ml of water are added, then the solution is evaporated. 200 ml of dichloromethane are added then the whole mixture is washed respectively 3x with a 0.1 N solution of aqueous HCl, 3 x with a brine solution then dried with Na_2SO_4, filtered, and the solvent is evaporated. 360 mg of a yellowish oil are obtained. The crude product is purified by chromatography on silica gel using 100 g of silica gel (0.04-0.0063 mm) and 1% of methanol in ethyl acetate as eluant. 230 mg (54%) of the pure derivative (5) are thus produced.

Cleavage of the MeBmt (OAc)-EtVal^4-Cs (5) acetate group and production of EtVal^4-Cs (6):

1.44 ml of a 0.45 molar solution of $NaOCH_3$ in MeOH (0.647 mmole) are added dropwise, under argon, to a solution

of 700 mg (0.562 mmole) of the derivative of Cs (5) in 28 ml of MeOH. [The solution of $NaOCH_3$ in methanol is prepared by adding sodium to pure MeOH.] The reaction is complete after 48 h with stirring at ambient temperature. The mixture is adjusted to pH 5 by adding 50% acetic acid in water. The solvents are removed under vacuum. The crude product is dissolved in 200 ml of ethyl acetate and extracted 2 x with water. The aqueous phase is re-extracted with 50 ml of ethyl acetate then the combined organic phases are washed 2 x with a brine solution, dried with Na_2SO_4, filtered and the solvent is evaporated.

The product obtained (750 g) is chromatographed on 180 g of silica gel (0.04-0.063 mm) using a solution of acetone/hexane 1:1 (20 ml fractions). 550 mg (82%) of ($EtVal^4$)Cs (6) are thus produced.

Preparation of H-EtVal-Ot-Bu:

4.1 ml (23.83 mmoles) of diisopropylethylamine are added, under argon, to a suspension of 5 g (23.8 mmoles) of H-ValOtBu x HCl in 1 l of trimethyloxoformate. At the end of 10 minutes the suspension becomes clear. 13.5 ml (0.24 mmole) of acetaldehyde dissolved in 30 ml of trimethyloxoformate are added dropwise to this solution under anhydrous conditions. The reaction mixture is stirred for 45 minutes under argon at ambient temperature.

Using a low vacuum, the excess acetaldehyde is removed by evaporation for 1 h 30. 25 g (0.112 mmole) of solid

$NaBH(OAc)_3$ are added, under argon, to this solution. After 15 minutes, the solution is cooled to 0°C and 500 ml of a 2% aqueous solution of HCl are added slowly.

The trimethyloxoformate is evaporated under vacuum and the remainder of the aqueous solution is diluted in 300 ml of water. This solution is then extracted 2 x with 100 ml of diethylether. The organic phase is then re-extracted 3 x with a 0.1 N aqueous solution of HCl. The combined aqueous phases are cooled to 0°C then the pH is adjusted to 9 using (2N)NaOH. The solution then becomes cloudy. The aqueous suspension is extracted 4 x with 100 ml of diethylether. The combined organic phases are then dried with Na_2SO_4, filtered and the solvent is finally evaporated.

4.2 g of a yellowish oil resulting from this step are purified by chromatography using 900 g of silica gel (0.04-0.063 mm) and a mixture of hexane/ethyl acetate 8:2 as eluant. Finally, 3.13 g (65%)of pure H-EtLeu-OtBu are obtained.

The results of Table 1 show the affinity of the derivatives of Cs for cyclophilin A in a competitive ELISA test described by Quesniaux in Eur. J. Immunology 1987, 17, 1359-1365. In this test, during incubation with cyclophilin, Cs bound to BSA (serum albumin) is added to the Cs to be tested. The concentration required to obtain 50% inhibition (IC_{50}) of the reference reaction in the absence of competitor is then calculated. The results are expressed by the binding index BI which is the ratio of the IC_{50} of the

derivative and the IC_{50} of CsA. A binding index (BI) of 1.0 indicates that the compound tested binds as well as CsA. A value lower than 1.0 indicates that the derivative binds better than CsA, and a value greater than 1.0 means that the derivative binds less well to CyP than CsA.

TABLE 1

Substance	Structure	BI	IR
UNIL 001	CsA	1.0	1.0
UNIL 002	$MeVal^4$-Cs	0.6	>200
UNIL 004	$EtVal^4$-Cs	1.0	>200
UNIL 007	$MeIle^4$-Cs	0.5	>200
UNIL 013	EtIle4-Cs	1.3	>200
UNIL 014	EtPhe(4-$CH_2PO(OMe)_2$)-Cs	0.5	>200

Cs is regarded as being immunosuppressive if its activity in the mixed lymphocyte reaction (MLR) is greater than 5%. The reaction (MLR) is described by T. Meo in "Immunological Methods", L. Lefkovits and B. Devis, Eds, Académie Prev. N.Y. pp: 227-239 (1979).

Spleen cells ($0.5.10^6$) originating from Balb/c mice (female, 8 to 10 weeks) are co-incubated for 5 days in the presence of treated spleen cells originating from CBA mice (females, 8 to 10 weeks). These cells were treated with mitomycin C or were irradiated at 2000 rads. The non-irradiated allogenic spleen cells exhibit a proliferative response in Balb/c cells which can be measured by incorporating a labelled precursor in the DNA. If the stimulator cells are irradiated (or treated with mitomycin C), the Balb/c cells no longer exhibit a proliferative

response but retain their antigenicity. The IC_{50} calculated in the MLR test is compared with the IC_{50} corresponding to CsA in a parallel experiment. The IR index is thus found, this being the ratio of the IC_{50} of the MLR test of the derivatives over the IC_{50} of cyclosporin A.

As with the binding index (BI) above, a value of 1.0 for the IR means an activity similar to CsA. Similarly, a lower value means better activity and a value greater than 1.0 shows that the activity of the compound is lower than that of CsA.

An IR value of > 20 shows that the substance is not immunosuppressive. The immunosuppression values of the derivatives are given in Table I.

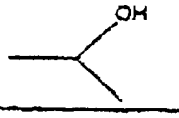
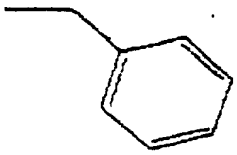
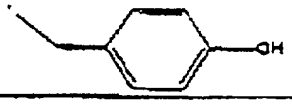
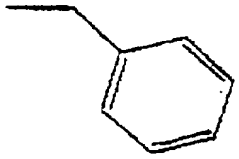
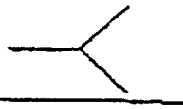
Table II describes the percentage protection during infection with HIV of a CEM-SS cell line. The protection of this line in the presence of a Cs derivative is compared with the infection of a line cultivated in the absence of Cs (reference control). A mean value is established at a concentration of the derivative of 2.10^{-6} molar. This anti-HIV activity was measured by the NCI (National Cancer Institute) in Washington in the USA.

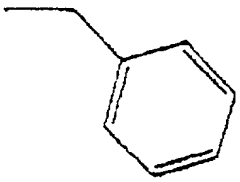
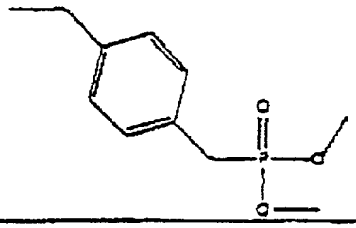
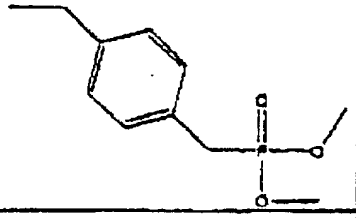
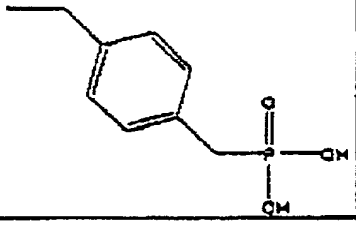
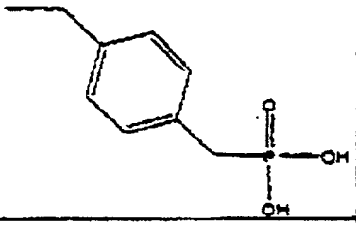
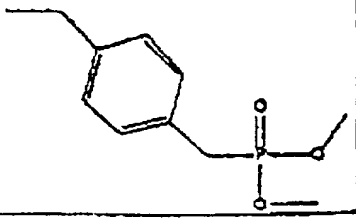
Table II

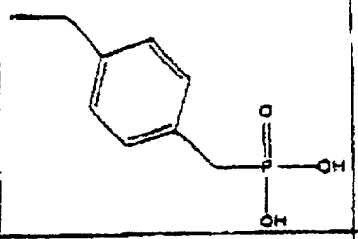
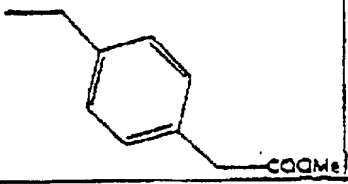
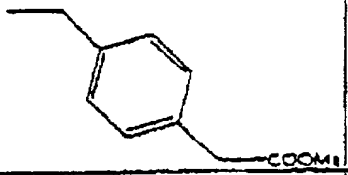
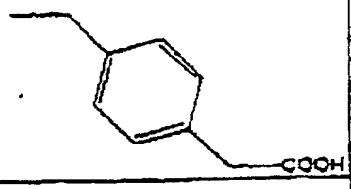
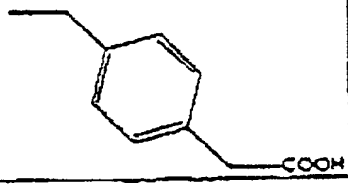
Substance	Structure	Percentage HIV Protection
UNIL 002	$MeVal^4$-Cs	66.4
UNIL 004	$EtVal^4$-Cs	74.9
UNIL 007	$MeIle^4$-Cs	68.5

A better percentage of protection against HIV infection obtained with the compound $EtVal^4$-Cs (compared with the two other references known to be 10 x better than CsA) shows the advantage of substitution by N-ethyl in position 4. This remark is even more pertinent if one compares the affinity for CyP of each substance. An affinity for CyP similar to that of CsA (BI = 1.0) is obtained for the $EtVal^4$-Cs derivative, whereas the derivatives $MeVal^4$-Cs and $MeIle^4$-Cs exhibit greater affinity for CyP (BI = 0.6 and 0.5 respectively). A greater anti-HIV activity corresponds to a lower affinity for CyP of $EtVal^4$-Cs. This clearly shows the value of this novel derivative.

TABLE III

Substance	R_1	R_2	R_3	R_4	$(\alpha)_D^{20}$
EtVal^{4}CS	$-CH_2CH_3$	CH_2CH_3	-H		c=0.07, MeOH -177
EtIle^{4}CS	$-CH_2CH_3$	CH_2CH_3	-H		c=0.05, MeOH -206
EtThr^{4}CS	$-CH_2CH_3$	CH_2CH_3	-H		
EtPhe^{4}CS	$-CH_2CH_3$	CH_2CH_3	-H		c=0.14, MeOH -159
EtTyr^{4}CS	$-CH_2CH_3$	CH_2CH_3	-H		
MePhe^{4}CS	$-CH_3$	CH_2CH_3	-H		c=0.06, MeOH -134
MeTyr^{4}CS	$-CH_3$	CH_2CH_3	-H		c=0.07, MeOH -95
D-MeAla^{3}EtVal^{4}CS	$-CH_2CH_3$	CH_2CH_3	$-CH_3$		c=0.12, MeOH -145
D-MeSer^{3}EtVal^{4}CS	$-CH_2CH_3$	CH_2CH_3	$-CH_2OH$		

Substance	R_1	R_2	R_3	R_4	$[\alpha]_D^{20}$
D-MeAla^3-EtPhe^4-CS	CH_2CH_3	CH_2CH_3	CH_3		c=0.06, MeOH -138.
D-MeAla^3-EtPhe^4(4-CH_2-PO(OMe)$_2$	CH_2CH_3	CH_2CH_3	$-CH_3$		
D-MeSer^3-EtPhe^4(4-CH_2-PO(OMe)$_2$	CH_2CH_3	CH_2CH_3	$-CH_2OH$		
D-MeAla^3-EtPhe^4(4-CH_2-PO(OH)$_2$	CH_2CH_3	CH_2CH_3	$-CH_3$		
D-MeSer^3-EtPhe^4(4-CH_2-PO(OH)$_2$	CH_2CH_3	CH_2CH_3	$-CH_2OH$		
EtPhe^4(4-CH_2-PO(OMe)$_2$	CH_2CH_3	CH_2CH_3	-H		c=0.05, MeOH -136

Substance	R_1	R_2	R_3	R_4	$[\alpha]_D^{20}$
Et^{4}Phe(4-CH_2-$PO(OH)_2$)	CH_2CH_3	CH_2CH_3	-H		
Et^{4}Phe(4-CH_2COOMe)CS	CH_2CH_3	CH_2CH_3	-H		c=0.15, MeOH -160
D-MeAla^3-Et^{4}Phe(4-CH_2COOMe)CS	CH_2CH_3	CH_2CH_3	$-CH_3$		
Et^{4}Phe(4-CH_2COOH)CS	CH_2CH_3	CH_2CH_3	-H		
D-MeAla^3-Et^{4}Phe(4-CH_2COOH)CS	CH_2CH_3	CH_2CH_3	$-CH_3$		

Claims

1) Synthesised cyclosporin characterised by the formula:

-X-U-Y-Z-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-
1 2 3 4 5 6 7 8 9 10 11

wherein:

X is -MeBmt or 6,7-dihydro-MeBmt-

U is -Abu, Nva, Val, Thr

Y is Sar or (D)-MeSer or (D)-MeAla or (D)-MeSer (OAcyl)

Z is (N-R)aa where aa={Val, Ile, Thr, Phe, Tyr, Thr (OAc), Thr (OG_1), Phe (G_2), PheCH_2(G_3), Tyr (OG_3)} with R = {alkyl > CH_3};

G_1 = {phenyl-COOH, phenyl-COOMe, phenyl-COOEt};

G_2 = {CH_2COOH, $CH_2COOMe(Et)_4$; $CH_2PO(OMe)_2$, $CH_2PO(OH)_2$};

G_3 = {$PO(OH)_2$, $PO(OCH_2CH{=}CH_2)_2$, CH_2COOH, $CH_2COOMe(Et)$}.

2) Cyclosporin according to claim 1, characterised in that the residue Z in position 4 is (R)Val where R>CH_3 and R<$C_{10}H_{21}$.

3) Cyclosporin according to any one of the preceding claims, characterised in that the residue Z in position 4 is N-ethyl-valine.

4) Pharmaceutical composition containing the compound characterised by the formula:

```
¦-X-U-Y-Z-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-¦
¦                                                ¦
¦ 1 2 3 4  5    6    7     8      9    10    11  ¦
--------------------------------------------------
```

wherein:

X is -MeBmt or 6,7-dihydro-MeBmt-

U is -Abu, Nva, Val, Thr

Y is Sar or (D)-MeSer or (D)-MeAla or (D)-MeSer (OAcyl)

Z is (N-R)aa where aa={Val, Ile, Thr, Phe, Tyr, Thr (OAc), Thr (OG_1), Phe (G_2), PheCH_2(G_3), Tyr (OG_3)} with R = {alkyl > CH_3};

G_1 = {phenyl-COOH, phenyl-COOMe, phenyl-COOEt};

G_2 = {CH_2COOH, $CH_2COOMe(Et)$, $CH_2PO(OMe)_2$, $CH_2PO(OH)_2$};

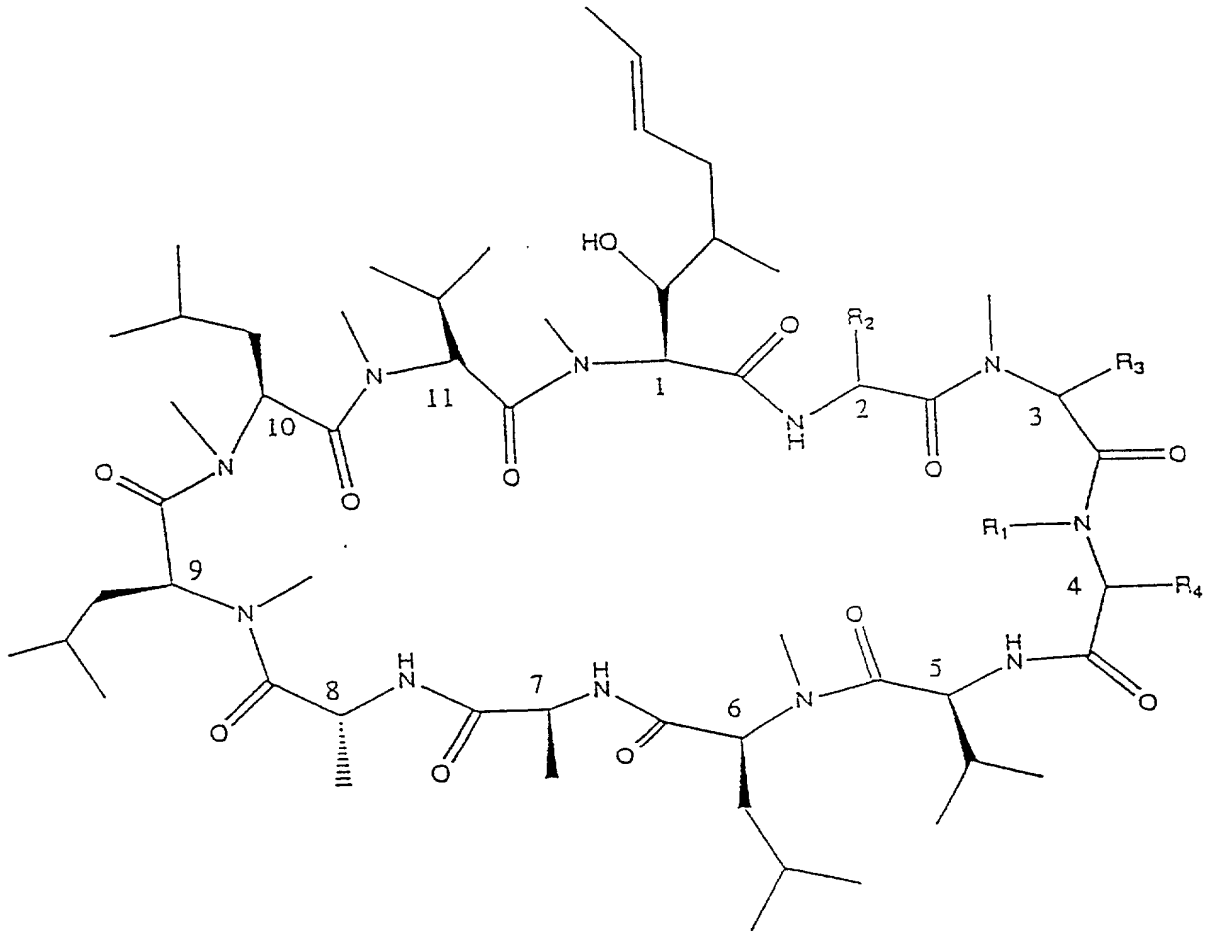
G_3 = {$PO(OH)_2$, $PO(OCH_2CH{=}CH_2)_2$, CH_2COOH, $CH_2COOMe(Et)$}

5) Pharmaceutical composition according to claim 4, characterised in that it is combined with a pharmaceutically acceptable solution.

6) Use of the cyclosporin according to any one of the preceding claims for the production of a medicinal product intended for the treatment and prevention of AIDS.

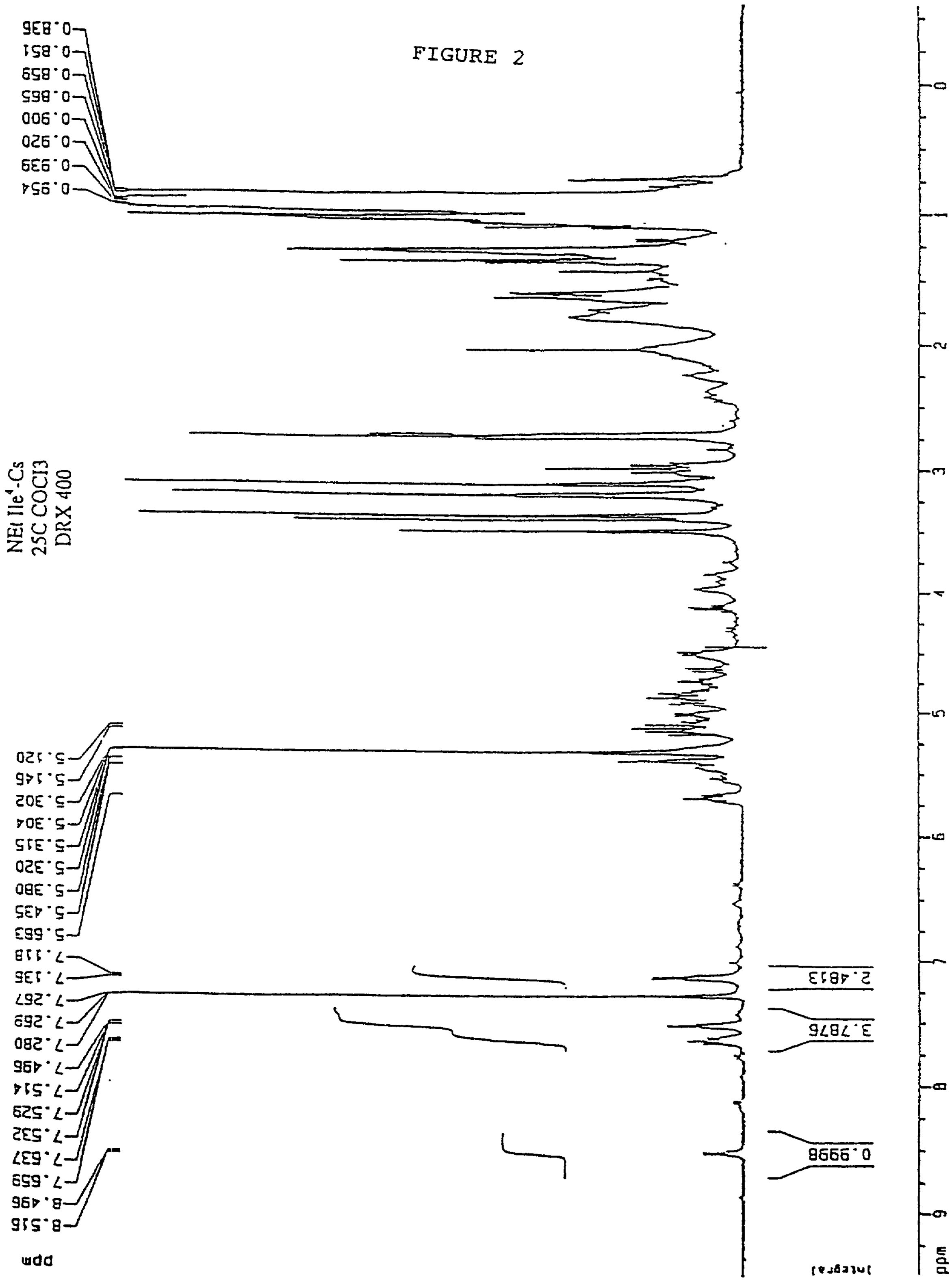
7) Use of the cyclosporin according to claim 3 for the production of a medicinal product intended for the treatment and prevention of AIDS.

FIGURE 1



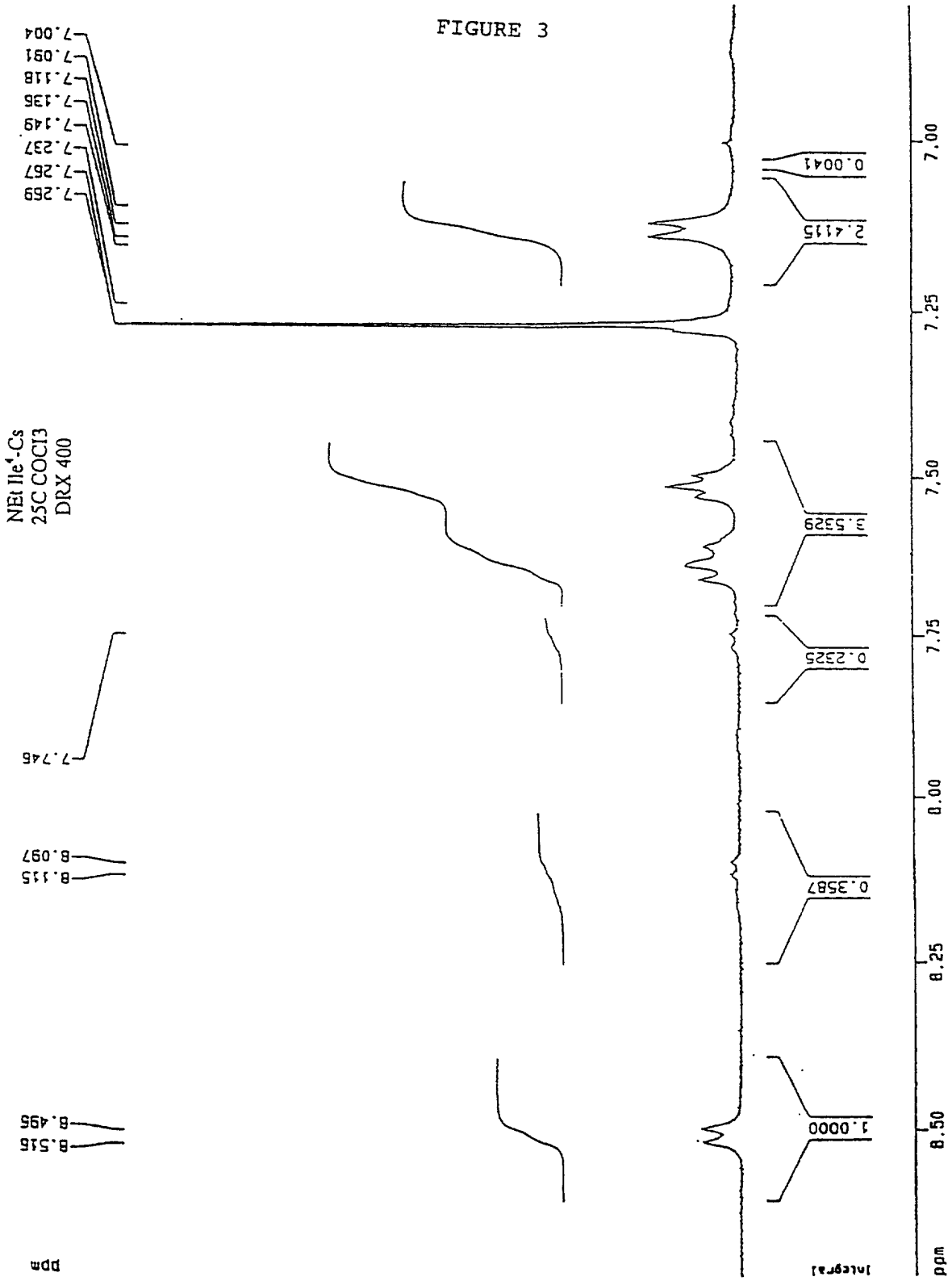
09/720923

FIGURE 2



SUBTITUTE SHEET (RULE 26)

FIGURE 3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integral
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7.25
7.50
7.75
8.00
8.25
8.50
ppm

c:\forms2\declarat.pct

US Nouvelle Cyclosporine

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY Docket No. 2-1032-150

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NOVEL CYCLOSPORIN HAVING AN IMPROVED ACTIVITY PROFILE, the specification of which

(check) ☐ is attached hereto.

☒ was filed on 28th December 2000 as Application Serial No. _____

and was amended on _____ (if applicable).

☐ was filed as PCT international application Number _____ on _____

and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me to be material to patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application (s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application (s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is now claimed:

Prior Foreign Application(s)			Priority Claimed
1405/98	SWITZERLAND	1th July 1998	X
(Number)	(Country)	(Day/Month/Year Filed)	Yes No
(Number)	(Country)	(Day/Month/Year Filed)	Yes No
(Number)	(Country)	(Day/Month/Year Filed)	Yes No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

IB99/01232	30th June 1999	
(Application Serial No.)	(Filing Date)	(Status--~~patented~~, pending, ~~abandoned~~)
(Application Serial No.)	(Filing Date)	(Status--patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agents(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: H. Robert Henderson, Reg. No. 18,486; Michael O. Sturm, Reg. No. 26,078; John E. Cepican, Reg. No. ~~26,851~~; Richard L. Fix, Reg. No. ~~28,297~~; William H. Wright, Reg. No. 26,424; Martin G. Mullen, Reg. No. ~~28,574~~; Daniel B. Greenwood, Reg. No. 35,885; and Curtis A. Bell, Reg. No. 36,742

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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